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Expression of transforming growth factor-β1 and β2 in rat glomeruli

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Expression of transforming growth factor-β1 and β2 in rat glomeruli. The transforming growth factors-β are potent modulators of cell growth and extracellular matrix metabolism in most types of cultured cells. The distribution and functions of TGF-β in vivo are less well known. We utilized several different techniques including northern blots, a CCl-64 cell growth inhibition assay, and sandwich enzyme-linked immunosorbent assays (SELISA) to examine the expression of TGF-β1 and TGF-β2 in rat glomeruli. High levels of TGF-β1 mRNA and protein were found in glomeruli (56 ± 22 ng TGF-β1/g tissue). These levels were several-fold higher than those present in whole kidney (10 ± 5 ng/g). TGF-β2 mRNA was present in glomeruli but was not detected in whole kidney. TGF-β2 concentrations by SELISA were 19 ± 8 ng TGF-β2/g in glomeruli and less than 5 ng/g in whole kidney. Since TGF-β has such marked effects on cell growth, we also examined whether alterations in TGF-β expression were associated with the renal hypertrophy which follows unilateral nephrectomy. Expression of TGF-β1 mRNA decreased in glomeruli following nephrectomy. However, this was not associated with a significant fall in glomerular TGF-β1 protein concentration. Whole kidney levels of TGF-β1 and its mRNA were unchanged following nephrectomy. Similar results were obtained for TGF-β2. Our data document the presence of high concentrations of TGF-β1 and β2 and their corresponding mRNAs in normal rat glomeruli. These results suggest that TGF-β may play important regulatory roles in the normal glomerulus.

The transforming growth factors-beta (TGF-β) are an expanding family of homologous peptides which have diverse effects on cultured cells. These effects have been most clearly elucidated for TGF-β1 and include, among others, regulation of cell proliferation and differentiation, and modulation of extracellular matrix metabolism [1, 2]. The distribution and roles of TGF-β in vivo are less well defined. TGF-β was found in tissues of mouse embryos during critical periods of morphogenesis, implying that an important role exists for TGF-β in embryogenesis [3]. TGF-β has also been shown to accelerate wound healing [4] and stimulate angiogenesis [5].

Based on the actions of TGF-β in vitro it seems reasonable to suspect that TGF-β might be important in normal tissues at sites where the regulation of extracellular matrix synthesis and cell proliferation are critical to the maintenance of normal organ function. The glomerulus represents one such site, however, recent studies using immunohistochemical techniques localized TGF-β in the kidney only to the distal tubules; no glomerular staining was found [6].

In the present study we examined the expression of both TGF-β1 and TGF-β2 in glomeruli. TGF-β1 and β2 are 25,000 molecular weight homodimeric peptides which share 71% homology and possess identical biological activity in most assays [2, 7]. At present the biological significance of these two forms of TGF-β are not known, however, the demonstration of preferential affinity of different receptors [8] for TGF-β1 and β2 suggest that unique roles may exist for these peptides.

We used a combination of northern blots, a CCl-64 growth inhibition assay, and sandwich enzyme-linked immunosorbent assays to assess levels of TGF-β1 and β2 and their mRNAs in normal glomeruli. These levels were compared with those present in whole kidney and renal papilla. Given the marked effects of TGF-β on the regulation of cell growth, we also evaluated the effect of unilateral nephrectomy on glomerular TGF-β expression

Methods

Animals/glomerular isolation

Animals utilized in these studies were male Sprague-Dawley rats weighing approximately 250 grams. Animals were allowed free access to standard rodent chow and water. At the time of sacrifice or nephrectomy animals were anesthetized with a subcutaneous injection of Innovar-Vet (Pitman-Moore, Inc., Washington Crossing, New Jersey, USA).

Nephrectomies were performed via a left flank incision using sterile technique. Glomeruli were isolated from the renal cortex which was dissected free from the remainder of the kidney, cut into small pieces and then gently pressed and rinsed through a 190 μm sieve (E-C Apparatus Corp., St. Petersburg, Florida, USA). After several additional passes through 190 μm sieves the glomeruli were isolated on top of a 74 μm sieve then gently pelleted (25 g for 2 min). These preparations contained > 90 to 95% glomeruli with < 5 to 10% tubular fragments.

Northern blots

Whole kidney or portions of renal cortex or papilla, which were dissected free from surrounding portions of the kidney,

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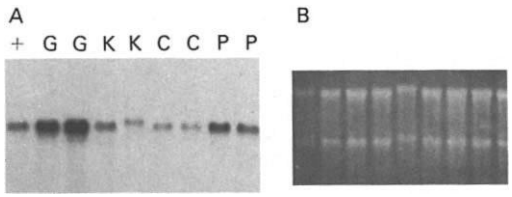

Fig. 1. *Expression of TGF-β1 in rat kidney.* Ten micrograms of total RNA was fractionated on 1% agarose formaldehyde gels, transferred to Nytran filters and probed with a single stranded TGF-β1 probe. **A.** +: 2 μg of total RNA from porcine lymphocytes used as a positive control. Abbreviations are: G, glomeruli; K, whole kidney; C, cortex; P, papilla. The autoradiograph was exposed for 3 days. **B.** Ethidium bromide stained gel used in panel A.

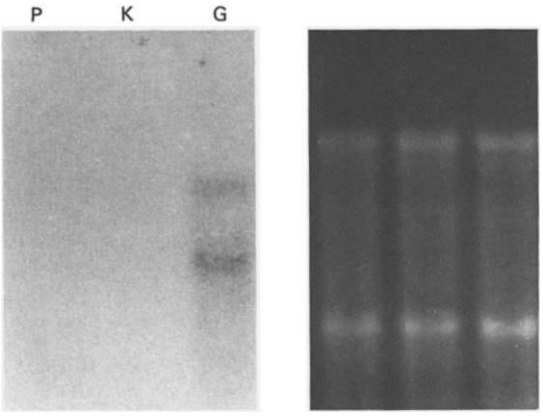

Fig. 2. *Expression of TGF-β2 in rat kidney.* Ten micrograms of total RNA from papilla (P), whole kidney (K), and glomeruli (G) were fractionated on 1% agarose formaldehyde gels transferred to Nytran filters and probed with a single stranded TGF-β2 probe. Two bands of approximately 4.5 and 3.5 kb were evident. The autoradiograph represents a 7 day exposure. The ethidium bromide stained gel used for this blot is also shown.

were homogenized in guanidine thiocyanate buffer and total RNA isolated as described [9]. Each whole kidney and papilla sample was derived from a single animal. Glomerular RNA was isolated from glomeruli pooled from eight to ten animals. RNA was quantitated based on its absorption at 260 nm. Ten micrograms of total RNA was fractionated on 1% agarose-formaldehyde gels and transferred to Nytran (Schleicher & Schuell, Keene, New Hampshire, USA). Hybridization conditions were those of Church and Gilbert [10]. The blots were hybridized in a solution containing 1% bovine serum albumin, 7% SDS, 0.5 M phosphate buffer pH 7.0, 10 mM EDTA, and 2 to 3 × 10^6 cpm of labelled probe for 16 to 20 hours at 65°C. Blots were rinsed three times for 10 minutes at room temperature in a solution of 0.5% BSA, 5% SDS, 40 mM phosphate buffer and 10 mM EDTA. Two or three stringent washes were then carried out using a solution containing 1% SDS, 40 mM phosphate buffer and 10 mM EDTA for 10 minutes at 65°C. Blots were exposed to Kodak X-Omat film for the indicated times, and then developed in a Kodak X-Omat processor.

Probes were prepared as follows. A single stranded probe of 218 nucleotides was made by Klenow extension using a specific oligonucleotide primer and ^{32}P alpha-dCTP [11]. Details regarding the construction of the M13 clone containing the human TGF-β1 cDNA are described by Van Obberghen and coworkers [12]. A single stranded probe from simian TGF-β2 cDNA [13] was made from an M13 construct having a portion representing both pro-region and the mature region of TGF-β2. The probe was made using a specific oligonucleotide primer and extending with Klenow fragment of DNA polymerase and ^{32}P alpha-dCTP, essentially as described [14]. A probe for glyceraldehyde phosphate dehydrogenase (pRGAPDH-13) [15] used as a control hybridization was made by random primer labelling method as described [16].

CCl-64 growth inhibition assay and SELISA

Kidneys were flushed free of blood then portions of whole kidney or renal papilla were weighed and homogenized (Tissuemizer: Teckmar, Cincinnati, Ohio, USA) in acid-ethanol (80% EtOH, 0.186 mM HCl, 50 μg/ml PMSF). Two milliliters of acid-ethanol was added to each 100 mg of tissue. Glomeruli were isolated as described above and then weighed and acid-ethanol added at the same weight/volume ratio. Kidney and glomerular preparations were extracted overnight with rotation at 4°C. The preparations were cleared by centrifugation and stored at −20°C until utilized. A CCl-64 mink lung epithelial cell growth inhibition assay was performed as described by Danielpour and co-workers [17], and as detailed in the legend to Figure 3.

For the SELISA, acid-ethanol extracts were lyophilized and then resuspended in 4 mM HCl, 0.1% bovine serum albumin. Preparation of the affinity-purified rabbit and turkey antibodies against TGF-β1 and TGF-β2 which were utilized in this SELISA are detailed by Danielpour and co-workers [18]. SELISAs for TGF-β1 and TGF-β2 were performed on these samples exactly as described [18], except that levamisole (2 mM) was added to the phosphatase substrate solution. Briefly, wells were coated with affinity-purified rabbit anti-TGF-β1 or TGF-β2, then washed in PBS containing 0.05% Tween 20, blocked with Tris buffered saline containing 1% bovine serum albumin, and washed again before test samples were added. Following a one hour incubation at room temperature with test samples, wells were washed and then coated with affinity-purified turkey anti-TGF-β1 or anti-TGF-β2. Plates were incubated for one hour at room temperature, washed, then incubated with phosphatase-linked goat anti-turkey IgG. Following this incubation plates were washed and phosphatase substrate in ethanolamine buffer with levamisole was added. The difference in absorbance at 410 and 450 nm was measured with a Dynatech MR600 ELISA reader. Background absorbance was determined for each sample in wells coated with pre-immune serum. TGF-β1 and β2 concentrations were determined by comparing the absorbance of samples (after subtracting background) to a log-log standard curve generated from absorbance readings of known concentrations of TGF-β1 and TGF-β2.

Results

TGF-β1 and β2 mRNA expression in normal rat kidney

Total RNA isolated from glomeruli, whole kidney, renal cortex, and papilla were used to examine the expression of TGF-β1 mRNA within the kidney. The 2.4 kb TGF-β1 transcript was found in all samples (Fig. 1). Glomeruli contained the highest steady state level of TGF-β1 mRNA. By scanning densitometry, levels were approximately four- to sixfold higher

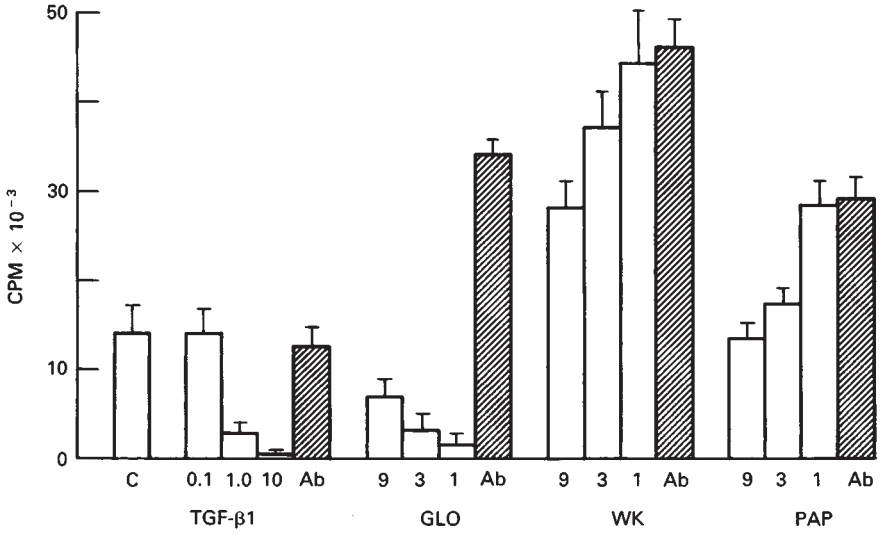

Fig. 3. *CCl-64 inhibition assay.* CCl-64 cells were plated in 500 μl of 0.2% FBS. Four hours later additions were made to the wells as follows: C:no additions, TGF-β1: 0.1, 1.0 or 10 pM TGF-β1, GLO: glomerular extract, WK: whole kidney extract, PAP: extract from renal papilla. Before addition to the culture wells the tissue extracts were diluted in PBS with 0.2% BSA. Fifty-five microliters of these dilutions were added to the culture wells to yield a final extract dilution of 1:100 (1), 1:300 (3), or 1:900 (9). The hatched bars labeled Ab indicate thymidine incorporation in wells to which TGF-β1 (10 pM) or tissue extracts (1:100) were preincubated for 30 minutes with TGF-β neutralizing antibody (120 μg/ml) (R & D Systems, Minneapolis, Minnesota, USA). After a 22 hour incubation, cells were labeled for 2 hours with ^{3}H-TdR, harvested, and counted. Results are expressed as the mean ± SD of preparations from three different animals. Each preparation was assayed in duplicate.

than those present in whole kidney. The renal papilla contained an approximately twofold higher level of TGF-β1 mRNA than was present in whole kidney. TGF-β2 mRNA, evidenced by two bands of approximately 4.5 and 3.5 kb, was present in glomeruli but was not detected in either whole kidney or renal papilla (Fig. 2).

CCl-64 cell growth inhibition assay

We first utilized a biological assay for TGF-β to search for TGF-β peptide in the kidney. In this assay acid-ethanol extracts of glomeruli, whole kidney, or renal papilla were tested for their ability to inhibit thymidine incorporation by CCl-64 cells, a cell line which is very sensitive to growth inhibition by TGF-β [17].

The addition of glomerular extracts to CCl-64 cells induced a reduction in their incorporation of thymidine in a dose dependent manner (Fig. 3). In contrast, thymidine incorporation in wells treated with glomerular extract preincubated with TGF-β neutralizing antibody was significantly greater than that present in control wells to which no additions were made. Addition of extracts from whole kidney or papilla resulted in an increase in thymidine incorporation which was minimally affected by preincubation of the extracts with TGF-β neutralizing antibody.

SELISA for TGF-β1 and TGF-β2

A SELISA using antibodies specific for TGF-β1 and TGF-β2 [18] was utilized to confirm the presence of TGF-β, to quantitate the peptide's tissue concentration, and to differentiate TGF-β1 and TGF-β2. Glomerular extracts contained high amounts of TGF-β1 (56 ± 22 ng/g tissue, mean ± SD, N = 7). Much lower concentrations (10 ± 5 ng/g tissue, N = 4) were found in whole kidney. Papilla contained an intermediate concentration of TGF-β1 (33 ± 13 ng/g tissue, N = 3). Tissue levels of TGF-β2 determined by TGF-β2 specific SELISA revealed 19 ± 8 ng of TGF-β2 per gram of glomerular tissue (mean ± SD, N = 4). Whole kidney and papilla contained less than 5 ng of TGF-β2 per gram of tissue.

Expression of TGF-β following nephrectomy

We first evaluated the effect of nephrectomy on TGF-β1 and β2 mRNA expression. As shown in Figure 4, levels of both

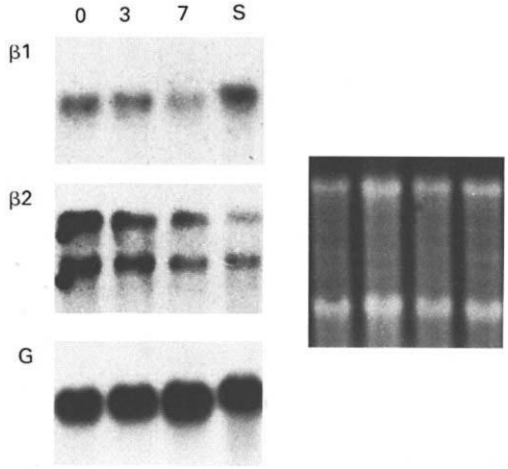

Fig. 4. *Glomerular expression of TGF-β1 and β2 and GAPDH mRNAs following nephrectomy.* Total RNA was isolated from rat glomeruli before (0), or three (3), or seven (7) days following nephrectomy. RNA was also isolated from rats seven days following sham nephrectomy (S). Samples were fractionated on a 1% agarose formaldehyde gel, transferred to a Nytran filter and hybridized with probes for TGF-β1 (β1), TGF-β2 (β2), and GAPDH (G). The filter was stripped after each hybridization. Results comparable to those presented were obtained in two independent experiments using different groups of animals. The TGF-β1 probed filter was exposed for 4 days, TGF-β2 for 6 days, and GAPDH for 16 hours. The ethidium bromide stained gel used for the above blots is also shown.

TGF-β1 and TGF-β2 mRNA in glomeruli decreased following nephrectomy. Of note is the decrease in TGF-β2 mRNA which also occurred in animals subjected to sham nephrectomy. In contrast to the findings for glomeruli, we detected no changes in TGF-β1 or β2 mRNA expression in whole kidney or renal papilla following nephrectomy or sham nephrectomy (data not shown).

Since the results of northern analysis indicated that glomerular TGF-β mRNA levels were lower at day seven than they were at earlier time points, we chose the seven day time point to assay TGF-β protein concentrations using the CCl-64 assay

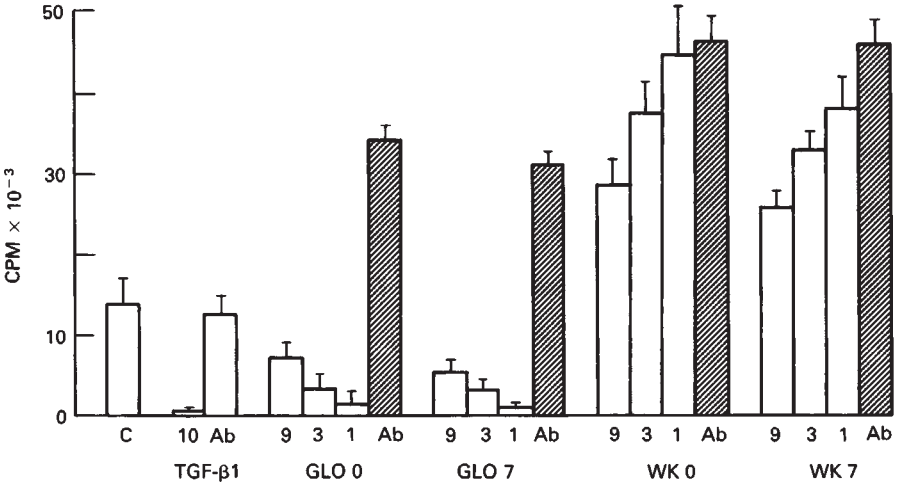

Fig. 5. *Effect of nephrectomy on biologic activity of acid ethanol extracts from glomeruli and whole kidney.* Acid-ethanol extracts from glomeruli (GLO) or whole kidney (WK) from unmanipulated rats (0) or those seven days following nephrectomy (7) were compared using the CCl-64 assay described in the legend to Fig. 3.

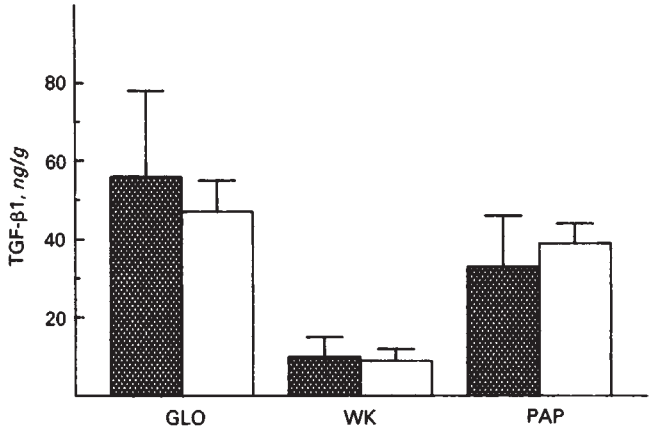

Fig. 6. *Effect of nephrectomy on TGF-β1 concentration.* TGF-β1 protein concentration was determined by SELISA on acid-ethanol extracted glomeruli (GLO), whole kidney (WK) and papilla (PAP) seven days after nephrectomy (open bars) or sham nephrectomy (hatched bars). Values are given as mean and standard deviation of the mean (GLO $N = 7$, WK $N = 4$, PAP $N = 3$).

and SELISA. We found no significant difference in thymidine incorporation in cells exposed to extract from glomeruli or whole kidney before or seven days following nephrectomy (Fig. 5). No significant differences in TGF-β1 protein concentration determined by SELISA were found when animals subjected to sham operations were compared to those subjected to unilateral nephrectomy (Fig. 6). We also examined TGF-β peptide levels by SELISA at eleven days following nephrectomy, reasoning that falls in protein levels might follow the decrease in TGF-β1 mRNA expression. Again we found no significant change in glomerular TGF-β1 levels eleven days following nephrectomy or sham operation (npx 47 ± 13, $N = 6$; sham 44 ± 0, $N = 3$). In addition, no difference in tissue levels of TGF-β2 in glomeruli, whole kidney and papilla were detected by TGF-β2 specific SELISA in nephrectomized, sham operated, and unmanipulated animals.

Discussion

In these studies we document the presence of high concentrations of TGF-β1 and β2 in normal rat glomeruli. We first searched for TGF-β expression in the glomerulus using northern analysis and found that glomerular TGF-β1 mRNA levels were approximately sixfold higher than those present in whole kidney. This result was somewhat surprising because recent studies using immunohistochemistry failed to detect TGF-β in the glomerulus [6]. We utilized two independent assays to demonstrate the presence of TGF-β in the glomerulus. The first, a CCl-64 growth inhibition assay, is an established biological assay for TGF-β [17]. The second assay was a SELISA for TGF-β1 and TGF-β2. The SELISA allowed us to differentiate TGF-β1 and TGF-β2 and to quantitate both peptides.

The concentration of TGF-β1 within the glomerulus (56 ng/g) is particularly impressive when considering the dissociation constants (K_d) of TGF-β1 receptors on cultured murine glomerular cells which range from 5 to 9 pM (0.125 to 0.225 ng/ml) [19]. While the precise functions of TGF-β within glomeruli are not defined, two potentially important roles include regulation of glomerular extracellular matrix metabolism and suppression of glomerular cell proliferation.

TGF-β increases the synthesis of collagen, fibronectin, and proteoglycans in a number of different cell types [1, 2]. In addition, TGF-β increases the accumulation of proteoglycans [20], collagen and fibronectin [19] by cultured glomerular mesangial cells, and increases the production of fibronectin by glomerular epithelial cells [19]. Given the marked effects of TGF-β on extracellular matrix synthesis in vitro and the high concentration of TGF-β in the glomerulus in vivo, it is possible that TGF-β plays a role in maintaining basal levels of extracellular matrix component synthesis within the normal glomerulus.

Another role for TGF-β in the glomerulus could be to regulate the proliferation of glomerular cells. We have previously demonstrated that TGF-β1 is a potent inhibitor of mouse glomerular endothelial, mesangial and epithelial cell proliferation [19]. TGF-β could act in vivo to inhibit glomerular cell proliferation, and thus play a role in the maintenance of the low rate of proliferation observed in normal glomeruli [21]. The potential importance of growth inhibitors such as TGF-β is suggested by our finding of potent mitogenic activity in extracts from normal glomeruli. It seems likely that the low rate of proliferation of glomerular cells in vivo may result from a careful balance between growth promoting and growth inhibiting stimuli. Stud-

ies are currently underway to characterize the mitogenic activity present in glomerular extracts.

The present study does not allow us to determine whether TGF-β in the kidney or glomerulus exists in the active or biologically latent form, since methods which effectively extract TGF-β from tissue sources (that is, acid-ethanol extraction) also serve to activate latent forms of TGF-β. Of interest, however, is the recent demonstration by Antonelli-Orlidge and coworkers [22] that co-cultures of bovine adrenal capillary endothelial cells and bovine aortic smooth muscle cells produce active TGF-β. When these cells are cultured alone they make a latent form of TGF-β. Glomeruli contain a similar combination of cell types. Within the glomerulus endothelial cells lie in close approximation to the smooth muscle cell-like glomerular mesangial cell [23]. Glomerular endothelial and mesangial cells could combine, as do bovine aortic smooth muscle cells and bovine endothelial cells, to produce active TGF-β within the glomerulus.

In this study we also demonstrated the presence of TGF-β2 in glomeruli. At present it is not known if TGF-β1 and β2 play different roles within the normal glomerulus. Studies to date have demonstrated equivalent biological activities of both peptides despite the preferential affinities of cellular receptors for the different TGF-βs [8]. We recently identified a unique group of binding proteins in rat glomeruli which bind TGF-β1 but do not bind TGF-β2 [24]. Whether these binding proteins play a role in defining differences in biological activity for the two peptides is not known.

Since TGF-β has such marked effects on growth we also examined the impact of unilateral nephrectomy on renal TGF-β expression. Nephrectomy altered TGF-β1 and β2 expression in glomeruli where marked falls in TGF-β mRNA levels occurred following nephrectomy. However, these falls were not associated with significant changes in glomerular TGF-β protein levels. The significance of the decline in glomerular TGF-β1 and β2 mRNA levels following nephrectomy is not known. The results could be explained if a large fraction of glomerular TGF-β is present in slowly turning over stores, or if a large fraction of glomerular TGF-β is derived from the circulation. If newly synthesized TGF-β has, for unknown reasons, greater bioactivity than that present in stores, then the falling TGF-β mRNA levels might indicate a permissive role for TGF-β in the glomerular growth which follows nephrectomy.

We [19] and others [20] have suggested that TGF-β might play a role in stimulating the accumulation of extracellular matrix material which occurs in the course of many progressive glomerular lesions. The finding of large concentrations of TGF-β1 and TGF-β2 in the normal glomerulus suggests, however, that TGF-β may have important regulatory functions under normal conditions as well. These functions could include the regulation of extracellular matrix metabolism which is so critical to the maintenance of normal glomerular function, and actions as an inhibitor of glomerular cell proliferation.

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